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β_2 integrin Mac-1 is a receptor for *Mannheimia haemolytica* leukotoxin on bovine and ovine leukocytes

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Abstract

Pneumonia caused by *Mannheimia haemolytica* is an important disease of cattle (BO), domestic sheep (DS, *Ovis aries*) and bighorn sheep (BHS, *Ovis canadensis*). Leukotoxin (Lkt) produced by *M. haemolytica* is cytolytic to all leukocyte subsets of these three species. Although it is certain that CD18, the β subunit of β_2 integrins, mediates Lkt-induced cytolysis of leukocytes, whether CD18 of all three β_2 integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CR4 (CD11c/CD18), mediates Lkt-induced cytolysis of BO, DS and BHS leukocytes remains a controversy. Based on antibody inhibition experiments, earlier studies suggested that LFA-1, but not Mac-1 and CR-4, serves as a receptor for *M. haemolytica* Lkt. PMNs express all three β_2 integrins, and they are the leukocyte subset that is most susceptible to Lkt. Therefore we hypothesized that all three β_2 integrins serve as the receptor for Lkt. The objective of this study was to determine whether Mac-1 of BO, DS and BHS serves as a receptor for Lkt. cDNAs for CD11b of BO, DS and BHS were transfected into a Lkt-non-susceptible cell line along with cDNAs for CD18 of BO, DS and BHS, respectively. Transfectants stably expressing BO, DS or BHS Mac-1 specifically bound Lkt. These transfectants were lysed by Lkt in a concentration-dependent manner. Increase in intracellular $[Ca^{2+}]_i$ was observed in transfectants following exposure to low concentrations of Lkt indicating signal transduction through secondary messengers. Collectively, these results indicate that Mac-1 from these three species serves as a receptor for *M. haemolytica* Lkt.

Keywords: *Mannheimia haemolytica*; Leukotoxin; Receptor; Mac-1; Cattle; Domestic sheep; Bighorn sheep

1. Introduction

Pneumonia caused by *Mannheimia haemolytica* is an important disease of cattle, domestic sheep and bighorn sheep (Foreyt and Jessup, 1982; Miller et al., 1991; Mosier, 1997; Brogden et al., 1998; Ackermann and Brogden, 2000). Leukotoxin (Lkt) secreted by *M. haemolytica* is the major virulence factor responsible for the pathogenesis of pneumonia caused by this organism. Lkt is a 102 kDa protein belonging to the RTX (repeats in toxin) family of pore-forming toxins produced by several Gram-negative bacteria (Lo et al., 1985; Welch, 1991). At high concentrations the toxin induces trans-membrane pore formation leading to the efflux of K^+, influx of Ca^{2+}, colloidal osmotic swelling, and eventual cytolysis (Clinkenbeard et al., 1989; Jeyaseelan et al., 2001). At sub-lytic concentrations, Lkt activates alveolar macrophages and PMNs resulting in the release of proinflammatory cytokines (Yoo et al., 1995) and induction of apoptosis (Stevens and Czuprynski, 1996).

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Previous studies in our laboratory and that of others identified β_2 integrins as the receptor for Lkt on the target cells (Wang et al., 1998; Ambagala et al., 1999; Li et al., 1999; Jeyaseelan et al., 2000). β_2 integrins are leukocyte-specific integrins, and have a common β subunit, CD18, which associates with three distinct α subunits, CD11a, CD11b, and CD11c, giving rise to three well-characterized integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CR4 (CD11c/CD18) (Gahmberg et al., 1997; Hogg et al., 2002). Recently identified β_2 integrin, CD11d/CD18 (Noti et al., 2000), has not been well characterized in ruminants. β_2 integrins are critical for leukocyte homing into areas of inflammation, phagocytosis, antigen presentation and cytotoxicity. Studies in our laboratory involving recombinant expression of CD18 in Lkt-non-susceptible cells have shown that CD18, the β subunit of β_2 integrins, mediates Lkt-induced cytolysis of leukocytes of cattle (Deshpande et al., 2002), BHS (Liu et al., 2007) and DS (Dassanayake et al., 2007). However, it is not clear whether the CD18 of all three β_2 integrins, LFA-1, Mac-1 and CR4, mediates Lkt-induced cytolysis of BO, DS and BHS leukocytes. Since PMNs, which express all three β_2 integrins, are the leukocyte subset that is most susceptible to Lkt, it is logical to hypothesize that all three β_2 integrins serve as the receptor for Lkt. LFA-1 of cattle, DS and BHS has been shown to be a receptor for Lkt (Jeyaseelan et al., 2000; Thumbikat et al., 2005; Lawrence et al., 2007; Dassanayake et al., in press). However, based on the results of antibody inhibition assays, earlier studies reported that Mac-1 does not serve as a receptor for *M. haemolytica* Lkt (Jeyaseelan et al., 2000; Thumbikat et al., 2005). The objective of this study was to determine unambiguously the role of Mac-1 in Lkt-induced cytolysis by developing transfectants expressing BO, DS or BHS Mac-1, and determine their susceptibility to Lkt-induced cytolysis without the confounding effects of LFA-1 and CR4.

2. Materials and methods

2.1. Cell lines and growth conditions

The human embryonic kidney cell line, HEK-293 (ATCC® Number: CRL-1573™) was cultured in complete culture medium (DMEM medium [Invitrogen] supplemented with 10% [v/v] heat-inactivated fetal bovine serum along with L-glutamine 4 mM and gentamicin 50 μg/ml [Sigma]). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. HEK-293 cells stably transfected with BHS and DS Mac-1 (CD11b/CD18) were selected and maintained in complete culture medium containing selection antibiotics: geneticin, 800 μg/ml (G418; Invitrogen) and blasticidin, 30 μg/ml (InvivoGen). BO Mac-1 transfectants were maintained on medium supplemented with 500 μg/ml each of hygromycin (Invitrogen) and geneticin.

2.2. Monoclonal antibodies and leukotoxin

The monoclonal antibodies (MAbs) specific for human CD11b (MM12A, IgG1), and human CD18 (HUH82A, IgG2a), which cross-reacts with BO, DS and BHS CD11b and CD18, respectively (Saalmuller et al., 2005) were obtained from Washington State University Monoclonal Antibody Center. The Lkt-neutralizing MAb MM601 (IgG1), and the FITC-conjugated Lkt-non-neutralizing MAb MM605 (IgG2a) developed earlier in our laboratory (Gentry and Srikumaran, 1991) were used in Lkt-neutralization and Lkt-binding assays, respectively. The MAbs 8G12 (IgG1) specific for bovine respiratory syncytial virus (Klucas and Anderson, 1988) and MM113 (IgG2a) specific for bovine herpesvirus 1 (Srikumaran et al., 1990) were obtained from the Department of Veterinary and Biomedical Sciences at the University of Nebraska-Lincoln, and used as isotype-matched controls. The Lkt from *M. haemolytica* (serotypes A1, A2 and A6) was prepared as described earlier (Gentry and Srikumaran, 1991). The culture supernatant containing Lkt was filter sterilized and stored at −20 °C in aliquots until needed. Same batch of Lkt was used in all the experiments.

2.3. Expression constructs of CD11b and CD18

The cDNA encoding bovine CD11b (Gopinath et al., 2005; GenBank Accession No. AY841169) was subcloned into the eukaryotic expression vector pcDNA3.1/Hygro(+) (Invitrogen) to yield the expression vector pWL/BO CD11b. The cDNA encoding CD11b from DS (GenBank Accession No. EF206308) and BHS (GenBank Accession No. EF206309) were cloned earlier into pUC19 vector (Lawrence and Srikumaran, 2007). In order to make expression constructs for transfection experiments, these genes were subcloned into mammalian expression vector, pcDNA6.2/GW/D-TOPO (Invitrogen) by PCR. All PCR reactions were carried out using PfuUltra™ II Fusion HS (Stratagene), a high fidelity DNA polymerase. The resulting constructs, pKL/DS CD11b and

pKL/BHS CD11b were used to transfect HEK-293 cells.

The cDNA for bovine CD18 (Shuster et al., 1992) obtained from Dr. Marcus Kehrli was subcloned into a eukaryotic expression vector pCI-neo (Promega) to yield the expression vector pMD/bCD18, in an earlier study (Deshpande et al., 2002). The cDNA encoding CD18 from DS (Dassanayake et al., 2007, GenBank Accession No. DQ470837) and BHS (Liu et al., 2006, Accession No. DQ104444) were cloned into mammalian expression vector pcDNA3.1D/V5-His-TOPO (Invitrogen) to yield pRD/CD18 and pWL/CD18 constructs respectively.

2.4. Co-transfection of cDNA for CD11b/CD18 into HEK-293 cells

In order to obtain BO Mac-1 transfectants, HEK-293 cells were co-transfected with pMD/bCD18 and pWL/BO CD11b using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. The BO Mac-1 transfectants were selected on medium containing 500 μg/ml each of geneticin and hygromycin. In order to obtain the ovine Mac-1 transfectants, purified plasmid DNA from pKL/DS CD11b and pRD/CD18 or pKL/BHS CD11b and pWL/CD18, were co-transfected into HEK-293 cells using TransFast™ as per manufacturer's protocol (Promega) with minor modifications. Briefly, HEK-293 cells at 60–80% confluency were harvested and seeded into 6-well tissue culture plates (5×10^5 cells/ml/well in complete growth medium) and incubated overnight. The following day, growth medium was aspirated and the cells were incubated with a mixture of 2 μg each of the plasmids diluted in 1 ml medium (serum free) along with TransFast™ reagent (charge to DNA ratio of 2:1). After incubation for 4 h at 37 °C, 1 ml culture medium containing 20% FBS was added into each well. Forty-eight hours post-transfection, cells were transferred into 75 cm^2 flasks containing selection medium (complete growth medium containing geneticin, 800 μg/ml and blasticidin, 30 μg/ml).

2.5. Magnetic cell sorting of transfectants

Transfected cell populations which continued to grow in the selection medium were subjected to cell sorting using MACS® system (Miltenyi Biotec) following the manufacturer's protocol. Briefly, the cells were incubated with anti-CD18 MAb (BAQ 30A) and anti-CD11b MAb (MM12A), followed by goat anti-mouse IgG MicroBeads and washed 3 times with 1× PBS (pH 7.2) buffer containing 0.5% BSA and 2 mM EDTA. The cells that bound to the MS column were eluted in DMEM medium and transferred to 75 cm^2 flasks containing selection medium. After 1 week of growth in the selection medium, the cells were pooled and analyzed for surface expression of CD11b/CD18 as described below. BO Mac-1 transfectants were sorted using fluorescence activated cell sorter (FACSVantage SE, BD Biosciences) into 96-well tissue culture plates to obtain single clones as described earlier (Lawrence et al., 2007).

2.6. Flow cytometric analysis of cell-surface expression of CD11b and CD18

Cell-surface expression of BO, DS or BHS CD11b/CD18 by the transfectants was determined by flow cytometric analysis according to previously published procedures (Deshpande et al., 2002). Briefly, 2.5×10^5 cells were resuspended in 50 μl of FACS buffer (3% horse serum and 0.01% sodium azide in 1× PBS) and incubated with 50 μl of anti-CD11b MAb MM12A, anti-CD18 MAb BAQ30A, or isotype-matched control MAb (15 μg/ml) at 4 °C for 20 min. Following three washes in FACS buffer, the cells were incubated with 50 μl of FITC-conjugated goat anti-murine Ig antibodies (Caltech Laboratories; 1:200 dilution) at 4 °C for 20 min. Similar treatment was given to the parent cells (HEK-293), which were used as the negative control. The cells were washed three times with FACS buffer, resuspended in 200 μl of PBS containing 2% paraformaldehyde and analyzed by a flow cytometer (FACSort, Becton-Dickinson Immunocytometry Systems).

2.7. Flow cytometric analysis of Lkt binding to BO, DS and BHS Mac-1

Lkt binding to the transfectants was tested by flow cytometry according to previously published procedures (Gopinath et al., 2005). Lkt binding to the transfectants was detected by FITC-conjugated MM605. The parent cells (HEK-293) were used as the negative control.

2.8. MTT dye reduction cytotoxicity assay for detection of Lkt-induced cytotoxicity

M. haemolytica Lkt-induced cytolysis of the transfectants was determined by a previously described

MTT dye reduction cytotoxicity assay (Gentry and Srikumaran, 1991). The parent cells (HEK-293) were used as the negative control. The percent cytotoxicity was calculated as follows:

$$\%\,\text{cytotoxicity} = \left[1 - \left(\frac{\text{OD of toxin} - \text{treated cells}}{\text{OD of toxin} - \text{untreated cells}}\right)\right] \times 100$$

2.9. Detection of Lkt-induced intracellular $[Ca^{2+}]_i$ elevation in transfectants expressing BO, DS and BHS Mac-1

Intracellular $[Ca^{2+}]_i$ elevation in transfectants expressing BO, DS and BHS and Mac-1 respectively was determined after exposure to Lkt. Ca^{2+} elevation was measured by fluorescent calcium indicator as described by the manufacturer (Molecular Probes).

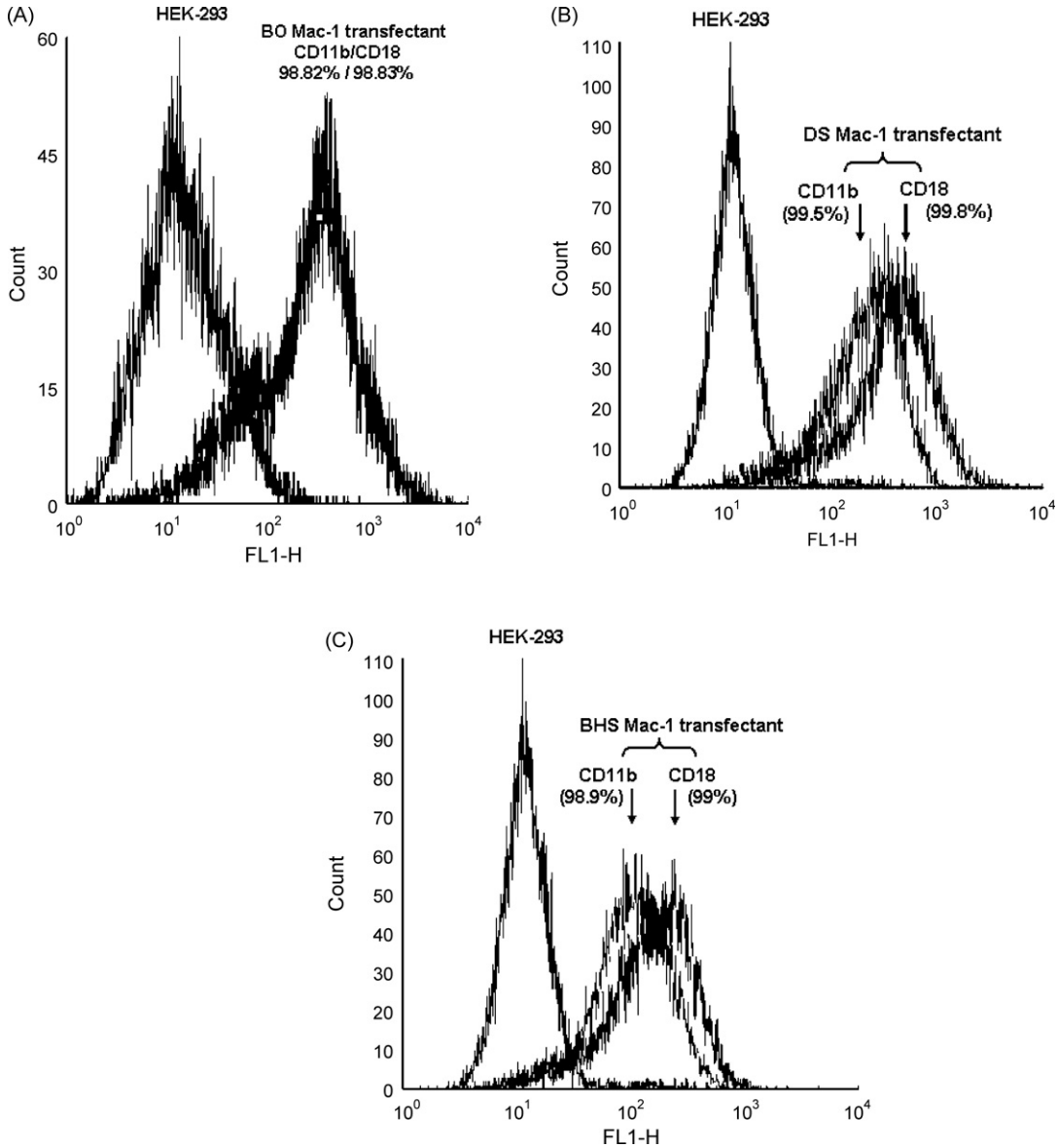

Fig. 1. The transfectants express BO, DS and BHS CD11b/CD18 (Mac-1) on the cell surface. The parent cells and the Mac-1 transfectants were stained with MAbs specific for CD11b (MM12A) or CD18 (HUH82A), followed by FITC-conjugated goat anti-murine Ig antibodies, and analyzed by a flow cytometer as described in Section 2. The figure shows fluorescence histogram overlays of parent cells (HEK-293), BO Mac-1 transfectants (A), DS Mac-1 transfectants (B) and BHS Mac-1 transfectants (C). Results of one representative experiment out of three are shown.

Culture supernatant from a *M. haemolytica* Lkt deletion mutant was used as the negative control (Murphy et al., 1995). Cells were then incubated with Lkt (40 U) for 60 s and intracellular $[Ca^{2+}]_i$ elevation was measured by flow cytometry. At least 5000 cells were counted to evaluate the intracellular $[Ca^{2+}]_i$ elevation.

2.10. Statistical analysis

Stastistical analysis was carried out using Student's *t*-test and *P* values were determined using http://www.graphpad.com/quickcalcs/ttest1.cfm (GraphPad Software). The term significant indicates a *P* value less than 0.05.

3. Results

3.1. Cell-surface expression of Mac-1 by the transfectants

Co-transfection of HEK-293 cells with plasmids encoding CD11b and CD18 of BO, DS or BHS resulted

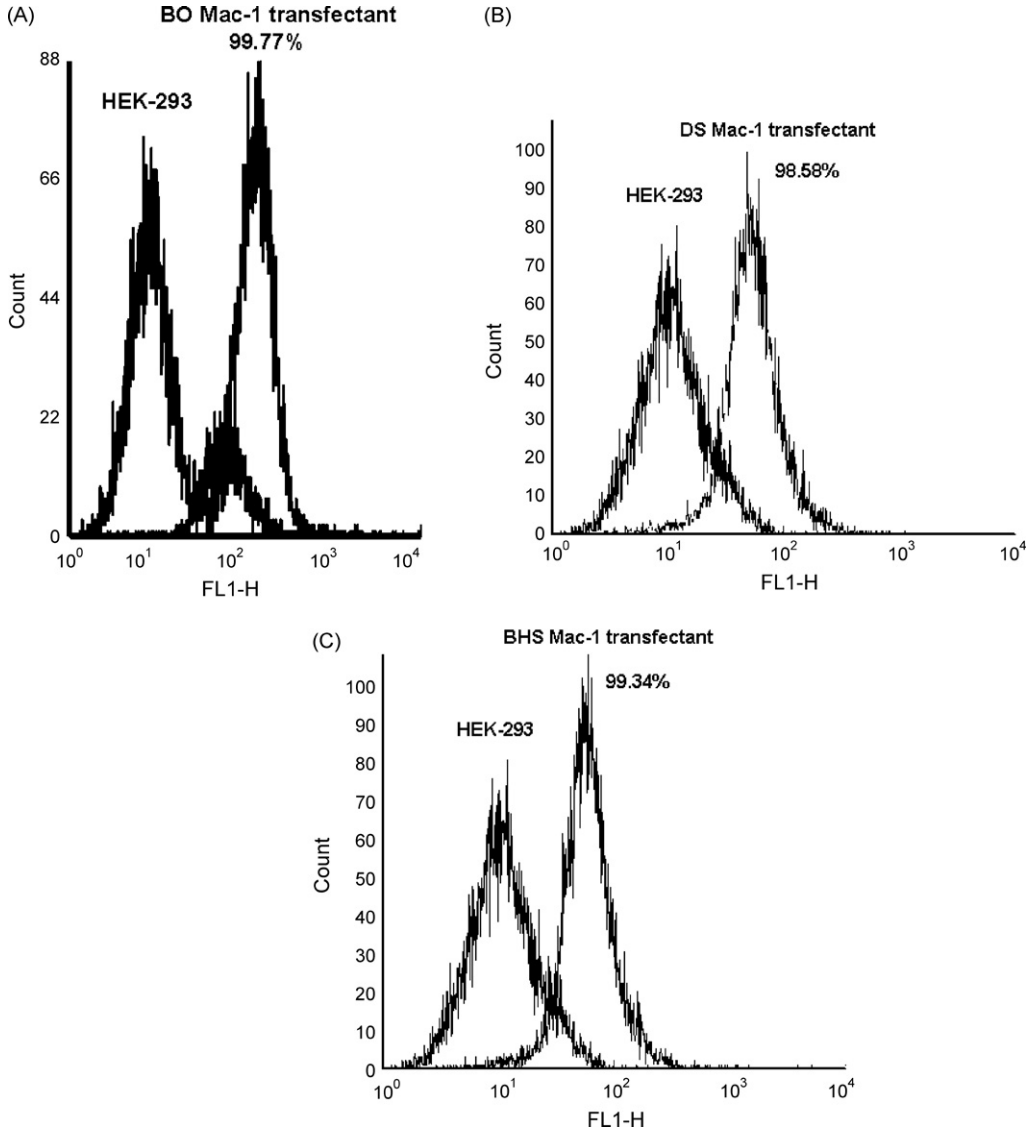

Fig. 2. *M. haemolytica* Lkt binds to transfectants expressing Mac-1. The parent cells (HEK-293) and the Mac-1 transfectants were incubated with Lkt, followed by FITC-conjugated anti-Lkt MAb, and analyzed by a flow cytometer, as described in Section 2. The figure shows fluorescence histogram overlays of parent cells (HEK-293), BO Mac-1 transfectants (A), DS Mac-1 transfectants (B) and BHS Mac-1 transfectants (C) treated with Lkt. Results of one representative experiment out of three are shown.

in cell populations resistant to the respective antibiotics. Three weeks following transfection, the transfectant cells were stained with MAbs specific for CD18 (HUH82A), and CD11b (MM12A), and subjected to magnetic sorting. Flow cytometric analysis of the sorted transfectants revealed that anti-CD11b and anti-CD18 MAbs, but not isotype-matched control MAbs, bound the transfectants confirming the cell-surface expression of BO, DS and BHS CD11b/CD18, respectively (Fig. 1A–C). These transfectants were designated as BO-Mac-1, DS-Mac-1 and BHS-Mac-1 transfectants respectively. In contrast to the DS-Mac-1 and BHS-Mac-1 transfectants, the BO Mac-1 transfectants enriched by magnetic sorting were not stable. Hence they were subjected to fluorescence-activated cell sorting to obtain single cell clones. One of these clones stably expressing BO Mac-1 (5G5) was selected for further analysis.

3.2. Leukotoxin binds to BO, BHS and DS Mac-1 transfectants

If Mac-1 serves as a receptor for Lkt, cell-surface expression of Mac-1 by BO-Mac-1, DS-Mac-1 and BHS-Mac-1 should enable Lkt to bind to them. Flow cytometric analysis of the transfectants after incubation with Lkt followed by FITC-conjugated anti-Lkt MAb MM605, revealed specific binding of Lkt to all three transfectants (Fig. 2A–C), but not the parent HEK-293 cells, suggesting that Mac-1 serves as a receptor for Lkt.

3.3. Lkt induces cytolysis of BO, DS and BHS Mac-1 transfectants

M. haemolytica Lkt has been reported to bind to cells that are not susceptible to Lkt-induced cytolysis (Sun et al., 1999). Hence binding of Lkt to the Mac-1 transfectants does not confirm the role of Mac-1 as a receptor for Lkt. Therefore, BO-Mac-1, DS-Mac-1 and BHS-Mac-1 transfectants and the parent HEK-293 cells were tested for susceptibility to Lkt-induced cytolysis by MTT dye reduction cytotoxicity assay. Lkt from the frequently isolated serotypes of *M. haemolytica*, A1, A2 and A6, lysed all three transfectants in a concentration-dependent manner (Fig. 3; the data from serotypes A2 and A6 are omitted from the figure for clarity). As expected the parent HEK-293 cells were not lysed by Lkt (Fig. 3). These results indicated that Mac-1 of all three species serves as a receptor for *M. haemolytica* Lkt. The Lkt-induced cytolysis of the transfectants was inhibited by pre-incubation of Lkt with Lkt-neutralizing MAb or pre-incubation of the transfectants with anti-CD11b or anti-CD18 MAbs, confirming that the cytolysis was induced by Lkt, and mediated by Mac-1 (data not shown).

3.4. Lkt induces intracellular $[Ca^{2+}]_i$ elevation in transfectants expressing Mac-1

Interaction of Lkt with the target cells results in the elevation of intracellular $[Ca^{2+}]_i$. This down stream

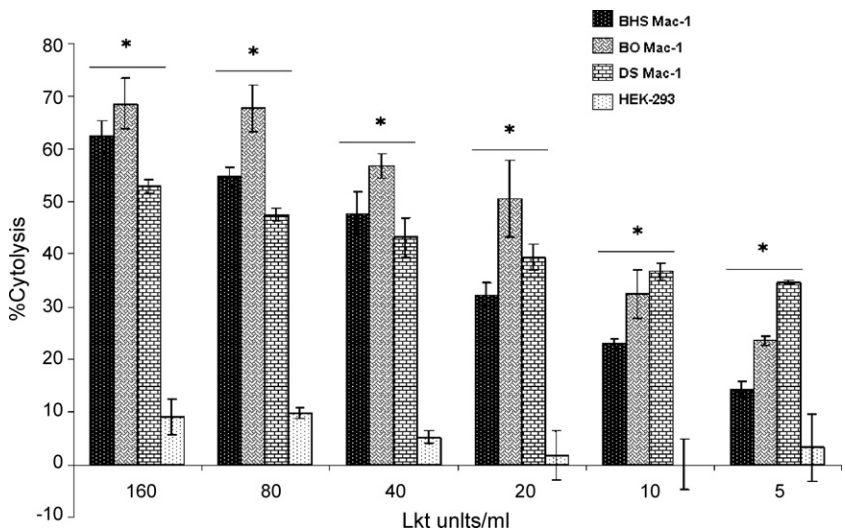

Fig. 3. *M. haemolytica* Lkt lyses Mac-1 transfectants in a concentration-dependent manner. The parent cells (HEK-293) and transfectants were incubated with varying concentrations of Lkt, and ensuing cytolysis was measured by the MTT dye reduction cytotoxicity assay, as described in Section 2. The transfectants, BO Mac-1, DS Mac-1 and BHS Mac-1 were lysed by Lkt in a concentration-dependent manner, but not the parent cells ($^{*}P < 0.0001$). Results shown are the means of three independent experiments.

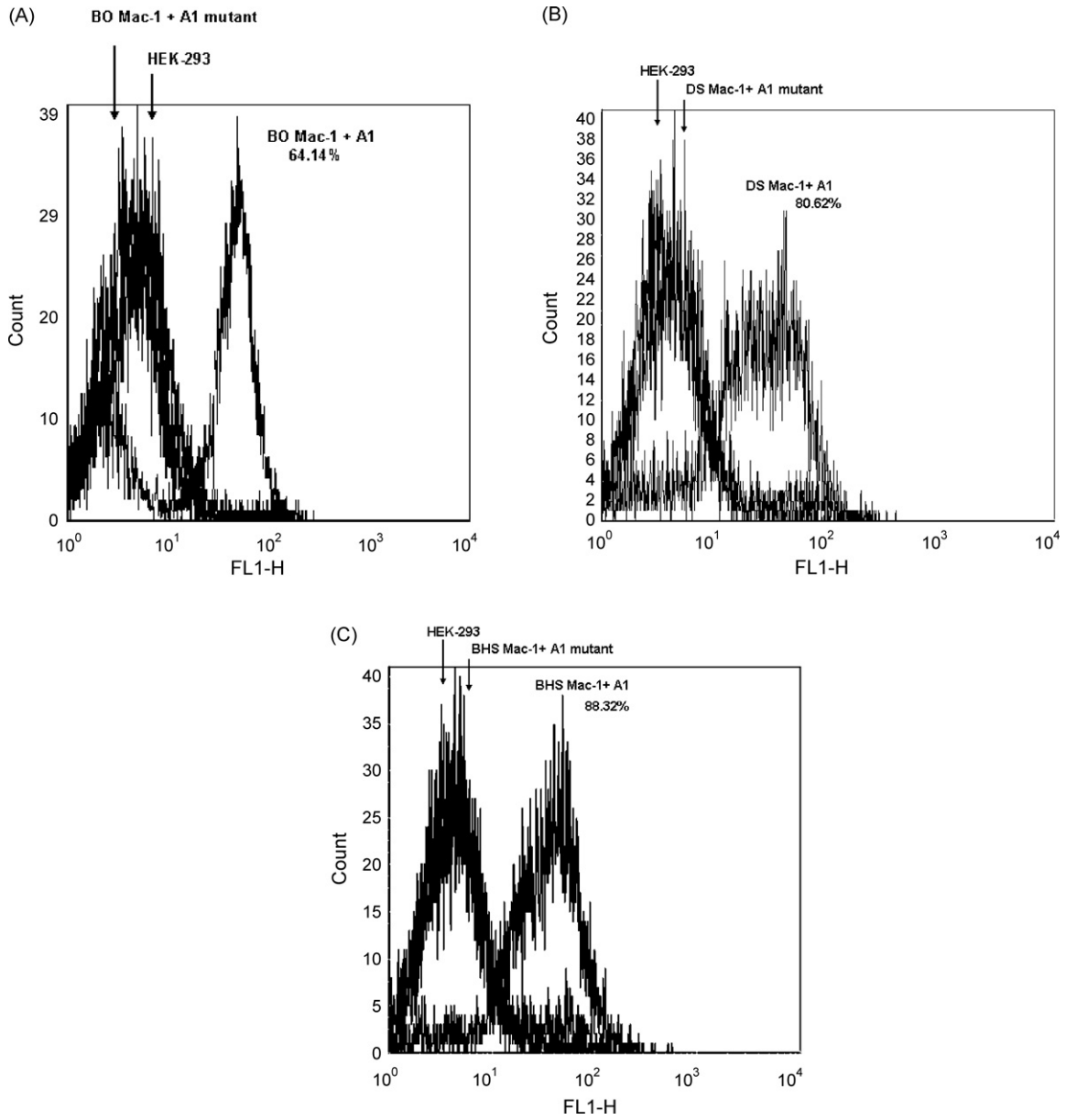

Fig. 4. *M. haemolytica* Lkt induces intracellular $[Ca^{2+}]_i$ elevation in Mac-1 transfectants. The transfectants and parent cells (HEK-293) were incubated with fluorescent calcium indicator (Fluo-4-AM) and exposed to 40 U of Lkt, and intracellular $[Ca^{2+}]_i$ elevation was analyzed by flow cytometry as described in Section 2. Lkt from the wild type *M. haemolytica* A1 induced intracellular $[Ca^{2+}]_i$ elevation in BO (A), DS (B) and BHS (C) Mac-1 transfectants, but not in HEK-293 cells. Intracellular $[Ca^{2+}]_i$ elevation was not observed in transfectant cells treated with culture supernatant from the mutant *M. haemolytica* A1. Results of one representative experiment out of three are shown.

event is an indicator of Lkt-receptor interaction (Dileepan et al., 2005; Thumbikat et al., 2005). Therefore, BO-Mac-1, DS-Mac-1 and BHS-Mac-1 transfectants were exposed to Lkt and intracellular $[Ca^{2+}]_i$ elevation was measured. Sixty seconds exposure to 40 U of Lkt was sufficient to elevate $[Ca^{2+}]_i$ levels in all three Mac-1 transfectants while Lkt had no effect on the parent cells (Fig. 4A–C). Culture supernatant from a Lkt deletion mutant of *M. haemolytica* had no effect, confirming that the intracellular $[Ca^{2+}]_i$ elevation is specifically induced by Lkt.

4. Discussion

M. haemolytica Lkt is cytolytic to ruminant leukocytes. We have previously demonstrated that the

β subunit of β_2 integrins, CD18, mediates Lkt-induced cytolysis of leukocytes of cattle (Deshpande et al., 2002), BHS (Liu et al., 2007) and DS (Dassanayake et al., 2007). The β subunit CD18 pairs with three different α subunits, CD11a, CD11b, and CD11c resulting in three distinct β_2 integrins, LFA-1, Mac-1 and CR4, respectively. It is not clear whether CD18 of all three β_2 integrins mediates Lkt-induced cytolysis. Since PMNs, which comprise the leukocyte subset that is most susceptible to Lkt-induced cytolysis, express all three β_2 integrins, we hypothesized that all three β_2 integrins serve as receptors for *M. haemolytica* Lkt. LFA-1 has already been shown to serve as a receptor in cattle, DS and BHS (Jeyaseelan et al., 2000; Thumbikat et al., 2005; Lawrence et al., 2007; Dassanayake et al., in press). However, two earlier reports suggested that LFA-1, but not Mac-1 and CR4, serves as the receptor for Lkt (Jeyaseelan et al., 2000; Thumbikat et al., 2005). Both of these studies, which are from the same laboratory, based their conclusion on the failure of anti-CD11b and anti-CD11c MAbs to inhibit Lkt-induced cytolysis of target cells and intracellular $[Ca^{2+}]_i$ elevation. Lack of inhibition of Lkt-induced cytolysis or intracellular $[Ca^{2+}]_i$ elevation by anti-CD11b and anti-CD11c MAbs does not necessarily mean that Mac-1 and CR4 does not serve as a receptor for Lkt. The inhibitory effect of a MAb depends on the location of the epitope recognized by the MAb relative to the location of the functional domain of the molecule. Furthermore, inhibition of binding of a ligand to its receptor by a MAb binding to a closely juxtaposed molecule has also been reported (List et al., 1999; Nason et al., 2001; Petersen et al., 2001). Therefore, inhibition of, or the lack of inhibition of, a biological effect of Lkt by a MAb could lead to invalid conclusions regarding the functional receptors of Lkt. Hence we resorted to the development of transfectants expressing Mac-1 to unambiguously determine its role as a functional receptor for Lkt. We chose the HEK 293 cell line for co-transfection of plasmids containing BO, DS or BHS CD11b/CD18 because of its lack of expression of β_2 integrins and the resultant non-susceptibility to Lkt-induced cytolysis. Flow cytometric analysis confirmed the cell-surface expression of CD11b and CD18 on all three Mac-1 transfectants (BO, DS and BHS). It has been established that the α and β subunits, CD11 and CD18, have to associate with each other in order to be transported to, and expressed on, the cell surface (Gahmberg et al., 1998). Histogram overlays from flow cytometric data (Fig. 1A–C) support the conclusion that CD11b/CD18 are expressed as a dimer on the cell surface of the transfectant clones.

Flow cytometric analysis indicated that Lkt specifically bound to Mac-1 transfectants, but not the parent cells, suggesting that BO, DS and BHS Mac-1 serve as a receptor for Lkt. Lkt binds to non-ruminant leukocytes also, but does not induce cytolysis (Jeyaseelan et al., 2000). But the concentration-dependent cytolysis of the transfectants by Lkt in our experiments clearly indicates that BO, DS and BHS Mac-1 serve as a receptor for Lkt. In general, *M. haemolytica* serotype 1 is commonly isolated from the pneumonic lungs of cattle and serotype 2 is commonly isolated from the pneumonic lungs of DS (Angen et al., 1999; Jeyaseelan et al., 2002). BHS are susceptible to both serotypes. In this study, BO, DS and BHS Mac-1 transfectants were equally susceptible to cytolysis by Lkt from all three serotypes, suggesting that Mac-1 can serve as a receptor for Lkt from these three different serotypes, and possibly others (data from *M. haemolytica* serotypes A2 and A6 not shown).

Following treatment with Lkt, intracellular $[Ca^{2+}]_i$ elevation occurs in target cells which is primarily due to the influx of extracellaular $[Ca^{2+}]_i$ through voltage-gated channels (Ortiz-Carranza and Czuprynski, 1992; Hsuan et al., 1998; Dileepan et al., 2005). Therefore, intracellular $[Ca^{2+}]_i$ elevation is considered as an indicator of Lkt binding to its receptor (Dileepan et al., 2005). In this study, Lkt induced intracellular $[Ca^{2+}]_i$ elevation in Mac-1 transfectants from all three species, but not in the parent cells, confirming the role of Mac-1 as a functional receptor for Lkt.

In summary, recombinant expression of Mac-1 on HEK-293 cell surface rendered them susceptible to binding and ensuing lysis by *M. haemolytica* Lkt, indicating that Mac-1 from BO, DS and BHS serves as a receptor for Lkt. Elevation of intracellular $[Ca^{2+}]_i$ in response to Lkt binding confirmed the role of BO, DS and BHS Mac-1 as a functional receptor for *M. haemolytica* Lkt. The Mac-1 transfectants enabled us to prove unambiguously that BO, DS and BHS Mac-1, like LFA-1, serves as a receptor for *M. haemolytica* Lkt. This finding directly contributes to the understanding of the differential susceptibility of the leukocyte subsets to *M. haemolytica* Lkt-induced cytolysis and the pathogenesis of this important disease of BO, DS and BHS.

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